STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS
THE BASIC FUCHSIN PROBLEM STILL WITH US

No stain has been studied longer and more intensively by the Stain Commission than basic fuchsin. Reports of progress on these investigations have appeared in Stain Technology on various occasions. The problem, nevertheless, is still unsolved and it sometimes seems as tho it is becoming more baffling the longer it is studied.

Recently certain complaints have been coming to us concerning certified batches of basic fuchsin for use in the staining of the tubercle organism. These complaints have caused some surprise because this has seemed a comparatively simple technic. The complaints, however, are to the effect that carbol fuchsin made up with some of these batches is either a gummy solution or precipitates upon standing. We have investigated all these complaints that have come in to us and have not as yet found them justified. Solutions of carbol fuchsin made up with all batches of certified stain seemed, in our laboratory, to be entirely satisfactory and reasonably permanent. We are, accordingly, at a loss to explain the complaints referred to.

Were this technic the only one for which basic fuchsin is employed the problem would be comparatively simple. There are, however, two other uses of this dye which present much more complicated problems—namely the Endo medium and the Feulgen stain. In both of these procedures, as has been explained in earlier reports, the dye, decolorized with sodium sulfite or bisulfite, is used as a chemical reagent. With the Endo medium the chemical reaction takes place in a culture dish, in the Feulgen stain it occurs in a microscopic section and is observed with the microscope. Neither reaction is thoroly understood. It is known that the color of fuchsin sulfurous acid is restored by aldehyde, but some other factor undoubtedly enters into both of these procedures. Thus in the Feulgen stain a simple restoration of the red is of no significance, as a true reaction should be purple; while on the Endo plates one expects certain organisms to restore the color without any surface luster, while the colon organism not only restores the red, but produces a decided metallic sheen.

No manufacturer is yet able to furnish consistently a basic fuchsin that is satisfactory for either of the reactions. Many batches of fuchsin submitted to us either failed to decolorize properly when made up either for the Endo or the Feulgen reagent, or else failed to show a proper color reaction upon use. There seems to be no correlation between the performance in this respect and the chemical composition of the dye itself. We are almost forced to the conclusion that the unsatisfactory results must be due to the presence of some impurity. What this impurity is, however, and how to get rid of it are still unsolved problems.

The uses for which basic fuchsin are most commonly purchased are for the Endo medium and for use in staining. Accordingly, the manufacturers refer to both of these uses on the label and samples submitted for certification are always tested for these two purposes before approval is granted. On account of the unsatisfactory tests above outlined, however, it has been necessary to approve many lots for use in the Endo medium which, altho satisfactory, are not as good as could be desired. This is unfortunate, but it is difficult to see how the Commission can be more strict in the matter when no information can be furnished to the stain companies as to how to improve their product.

In regard to the Feulgen test, the situation is different, inasmuch as fuchsin is seldom ordered for this purpose and the manufacturers have not yet mentioned the technic on their labels. Accordingly, altho the companies are trying to get a good fuchsin for this purpose, and altho all samples submitted are tested in the Feulgen procedure, neither the manufacturers nor the Stain Commission can give any assurance that certified samples will be satisfactory when thus employed. Neither the Endo nor the Feulgen problem will be solved until we learn the cause of the trouble and how to produce uniformly a satisfactory product.

H. J. Conn

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the October number of this journal.

Stains Certified September 1 to November 30, 1932*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Toluidine blue O	CU 2	52%	For general histological staining	Sept. 2, 1932
Light green SF yellowish	CL 3	79%	For general histological staining	Sept. 2, 1932
Anilin blue W.S.	CK 3	....	For general histological staining	Sept. 2, 1932
Brilliant green	LBg 1	96%	For use in bacteriological media	Sept. 2, 1932
Methyl violet	NMv 3	88%	For histological and bacteriological staining	Sept. 9, 1932
Gentian violet	CB 9	80%	For histological and bacteriological staining	Sept. 9, 1932
Sudan III.....	LY 1	70%	As fat stain	Sept. 10, 1932
Methyl green	CG 5	60%	For general staining and in Pappenheim stain	Sept. 23, 1932
Methylene blue	CA 14	85%	As bacteriological and histological stain	Sept. 27, 1932
Erythrosin	CEr 2	83%	As histological stain	Sept. 27, 1932
Eosin Y	CE 6	88%	For general histological staining	Oct. 4, 1932
Hematoxylin....	FH 10	....	For general histological staining	Oct. 8, 1932
Neutral red	CX 4	50%	For histological and vital staining	Oct. 11, 1932
Nigrosin	LNi 2	....	For histological and bacteriological staining	Oct. 15, 1932
Basic fuchsin	NF 21	85%	For staining and in the Endo medium	Oct. 27, 1932
Carmin	CCa 3	....	For general histological staining	Nov. 10, 1932
Orange G	CO 4	90%	For general histological staining	Nov. 15, 1932
Wright's stain	NWr 8	....	As blood stain	Nov. 23, 1932

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

THE HISTORY OF STAINING

H. J. Conn, *Geneva, N. Y.*

SIR JOHN HILL

Dr. John Hill is of interest to the modern histologist in spite of the fact that he died before the beginning of the nineteenth century and made very few contributions to actual histology. The histologist's interest in his work is due largely to the fact that he seems to have been the first to use a dye in coloring material to be examined under the microscope. His contribution to this field is described elsewhere in this series of papers.

Sir John Hill was born in 1716 or 1717, son of the Reverend Theophilous Hill who was located at that time either at Peterborough or Spalding, England. He started out in life with the apparent object of practicing medicine and is first heard of as an apprentice to an apothecary. This line of work, however, was given only a minor amount of his attention and all of his chief contributions were made in other fields. He was indeed a very versatile writer and contributed to nearly every branch of science, as well as issuing a series of essays on all phases of contemporary life, and making contributions to fiction, the classics, theology, and history.

In one early period of his life he tried to become an actor and was so poorly impressed with the theater of his day that he promptly published a treatise on the art of playing, in which he criticised actors and theater managers so severely as to stir up a rather bitter quarrel.

Between these various ventures, however, his chief interest always remained botany. In his early twenties he collected plants thruout England, dried them and arranged them in sets with classifications and descriptions. These herbaria he sold when he could, and thus attempted to add to his income. He was, in the meantime, becoming much interested in making new contributions to botany and published more extensively in this field than in any other.

In his scientific contributions, unfortunately, he sometimes went beyond the actual facts and thus laid himself open to criticism. His contemporaries were very ready to point out such mistakes, as he himself was highly critical of others. His early quarrel with the theatrical profession was small in comparison with the one which he later provoked with the Royal Society. Undoubtedly, many of his criticisms were fair and many of his opponents criticised him unfairly. Nevertheless, there can be no question but that he was too

SIR JOHN HILL
1716 (or 17)–1775

ready to stir up a fight and had much less influence in his chosen field than he would have had if he had used more conciliatory methods.

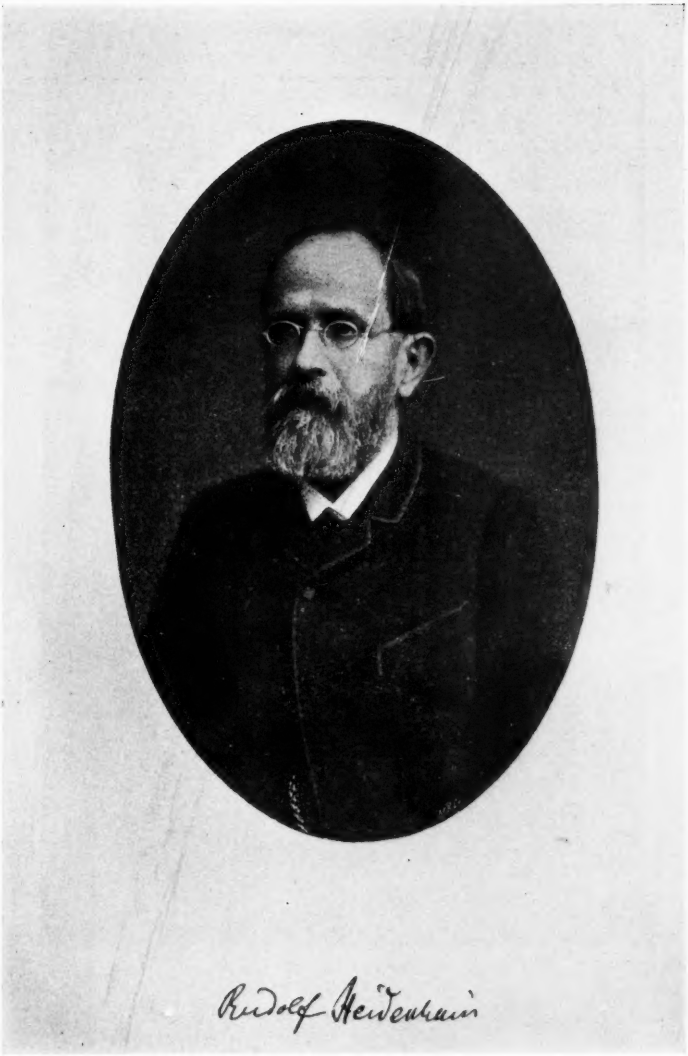
Among his various contributions to botany was the pamphlet on the construction of timber in which he described the method of making microscopic sections of stems which he had placed in a solution of carmin to allow the dye to ascend thru the vessels. This paper can hardly be considered one of his greatest pieces of work, but his use of carmin in this study makes him, beyond much question, the earliest pioneer in the use of dyes for microscopic purposes.

RUDOLF HEIDENHAIN

Dr. Rudolph Heidenhain was born January 29, 1834, at Marienwerder in West Prussia. He was the son of a prominent physician and was thus led naturally into the medical profession. He had five brothers, moreover, all of whom entered some medical field. He was educated in this line, studying in Berlin under Johannes Müller and E. Du Bois-Reymond where he specialized in physiology. He took his advanced work at Halle under Dr. A. W. Volkmann, whose daughter he married. He wrote a thesis on the determination of the quantity of blood in the body.

When 26 years old he became Professor of Physiology and Director of the Physiological Institute at Breslau. Besides work in physiology itself, he conducted a course in microscopy and had charge of work in microscopic anatomy, in both of which fields he made outstanding contributions. At the time when he received his appointment at Breslau (1859), Gerlach had just made his discovery as to how to use carmin for histological work without overstaining. It was natural that Heidenhain should begin using staining procedures in his work. Hematoxylin, as well as carmin, was coming into use at about the same period as shown by the publication of Böhmer's formula for alum-hematoxylin in 1865. It is plain that Heidenhain experimented with this dye and tried other mordants besides alum, for in 1885 he proposed a chrom-hematoxylin which led the way to various more modern modifications that are still being used to-day. This formula, however, must not be confused with what is to-day ordinarily called Heidenhain's hematoxylin, which is the iron formula proposed by his son, Martin Heidenhain.

R. Heidenhain's most important work was on the subject of secretions and alimentary absorption, in connection with which he published a large monograph "Beiträge Zur Histologie und Physiologie der Dünndarmschleimhaut." He also made contributions to the physiology of muscles, nerves and the brain. He was an inspiring teacher and made so many followers among his students that his name is due to his scholars' work fully as much as to his own contributions. He died in October, 1897, at the age of sixty-four years. His son, Dr. Martin Heidenhain, Professor of Anatomy at Tubingen, represents the third generation of the family to make outstanding contributions in medicine.



RUDOLPH HEIDENHAIN
1834–1897

THE BIELSCHOWSKY STAINING TECHNIC

A STUDY OF THE FACTORS INFLUENCING ITS SPECIFICITY FOR NERVE FIBERS

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Abstract.—**By comparing results obtained with adult mammalian tissue from introducing variables into each separate step in block-staining by the Bielschowsky silver method, the following conclusions were reached:**

1. No specific means for inhibiting the staining of connective tissue and still permitting complete staining of nerve fibers was found, but the avoidance of overstaining was very helpful toward such differentiation.

2. Overstaining could be corrected by reducing the concentration of the silver nitrate bath or by adding an excess of ammonia to the ammoniated silver bath.

3. Staining of fine fibers was favored by adding acetic acid to the formaldehyde used for fixation or by adding pyridin to the silver nitrate bath.

4. Addition of protein-precipitating organic acids (trichloracetic or sulfosalicylic) to the fixative was disadvantageous.

5. Prolonged fixation favored an increase in intensity of the stain. Four days' time was sufficient.

6. Extraction of lipids with ammoniated alcohol gave results similar to those obtained after extraction with pyridin, but the stain was lighter.

7. Ammoniated silver carbonate without excess ammonia had an action similar to ammoniated silver hydroxide with excess ammonia.

8. An excess of ammonia in the ammoniated silver solution (Ag 0.1 N) was tolerated, without apparent impairment of nerve-fiber staining, up to 6 M NH_3, altho the use of more than 3 M excess (2 cc. concentrated ammonia water added to 100 cc. of balanced ammoniated silver hydroxide solution) seemed unnecessary.

9. Impregnation with 1.7% (0.1 N) silver nitrate solution was quite satisfactory and variations in the concentrations of this bath suggested that the practical limits of concentrations that would be generally satisfactory lay between 0.3 and 3.0%.

10. The writers' experiences agreed with Agduhr's relative to the advantage of washing in 2.5% acetic acid between the ammoniated silver bath and formaldehyde reduction.

[1]Contribution No. 180. Presented as a thesis by R. H. Beech in partial fulfilment of requirements for the degree of Master of Science.

Introduction

Altho Simarro (1900) published the first work on the impregnation and subsequent reduction of silver salts in nervous tissue, his methods were too complicated for general application. A few years later, Cajal and Bielschowsky introduced simpler procedures which are still used.

The present work was undertaken to study the Bielschowsky method as applied to nerve cells and their processes by subjecting the various steps in the process to variation. It was hoped to differentiate the more critical from the less critical variables in the technic and thereby enable the inexperienced or previously unsuccessful worker to obtain a larger percentage of successful preparations.

Historical

Robertson's early work dated from 1898 when he began to work on a method for using the reducing action of formaldehyde on ammoniated silver in staining nervous tissue. Fajerstajn (1901) was also among the first to use the reaction of silver nitrate and ammonia for the staining of nervous tissue. In his original method he used sections of tissue which had been fixed in formaldehyde and cut with a freezing microtome. The results obtained seem to have been histologically usable, but appear inconstant, since he stated that the method had its faults and needed further perfection.

Bielschowsky (1902) first presented the method which now bears his name. His formula varied from that of Fajerstajn, by making use of an extra formaldehyde bath after cutting the sections, and 10% instead of 2% silver nitrate in making up the ammoniated silver bath. He improved the gold toning process by treating the toned sections with 10% sodium thiosulfate to remove the silver chloride which remained after the gold bath. This preliminary work was extended (Bielschowsky, 1904a) and a definite improvement over that of Fajerstajn's was made. The improvement consisted of impregnating the material for 24 hours in a 2% solution of silver nitrate before treatment with the ammoniated silver solution. A further modification of the technic was introduced later in 1904. This consisted of staining sections a few minutes longer in the ammoniated silver bath until they had acquired a decided brown color and then washing them in 10 cc. of distilled water acidified with five drops of acetic acid. In this solution the sections acquired a yellowish tinge, sometimes in a few seconds. Reduction was accomplished in an 8% instead of a 4% formaldehyde solution. In addition, Bielschowsky stated that he was able to differentiate myelinated from unmyelinated fibers by soaking the sections in a copper sulfate solution before placing them in the silver bath.

In 1909 he published the method which has been used as the standard in this investigation. The addition of the pyridin treatment before impregnation constituted the final refinement. He attributed the use of pyridin to a suggestion made by Held (1907) and stated that the pyridin caused more selective staining, so that the finest nerve fibers were brought out and the connective tissue was unstained, and that it dissolved out the myelin so that the myelinated fibers were stained darker.

Wolff (1905), in trying to determine whether neurofibrils ended on the cells as end bulbs or continued as a synctium, used the method of Bielschowsky in preference to that of Cajal. He noted that prolonged washing was necessary after fixation in order to prevent too dark a stain and to insure some differentiating effect.

Favorsky (1906) used the Bielschowsky method for investigating the structure of the olfactory bulb. His modifications of the method consisted of the use of 8% formaldehyde for fixation, 10% silver nitrate instead of 2% for impregnation, and 0.1% gold chloride solution for gold toning.

Paton (1907) advocated the use of 0.75% to 1% silver nitrate for the preliminary silver bath. As in one of Bielschowsky's earlier methods, he washed the sections in a solution of 10 cc. of water acidified with 5 drops of glacial acetic acid following the ammoniated silver impregnation. As a reducing solution, he used 20 cc. of 1% hydroquinone with 2 cc. of neutral formaldehyde solution.

Schutz (1908) soaked his material in water before making frozen sections; soaked the sections after sectioning for 2 to 3 hours; and soaked them again for 24 hours after the preliminary silver bath. Thus, he indicated that the amount of washing hitherto used had been insufficient. He used an acetic acid bath (composed of 2 drops of glacial acetic acid in 10 cc. of distilled water) after formalin reduction as an adjunct to the gold toning procedure.

Boeke (1909) fixed material in a solution of 4% formaldehyde in 60% alcohol and used several changes. He found that a long fixation time was necessary in this alcohol-formaldehyde mixture, and that the material suffered no damage if left in it for exceedingly long periods. When the blocks were to be used, he transferred them to 4% formaldehyde until they were free from alcohol. The succeeding steps followed Bielschowsky's method with or without pyridin extraction.

Schlemmer (1910) found that the greatest source of error lay in making up the ammoniated silver solution, and modified the procedure to correct this fault. Sodium hydroxide was added to 10% silver nitrate until it caused no further precipitate. After allowing the precipitate to settle completely, he poured off the supernatant fluid, filled the flask with distilled water, and repeatedly washed the precipitate until the wash water was no longer alkaline. He then dissolved the precipitate with the smallest possible amount of ammonia and filtered the concentrated solution thru glass wool. This solution was found to be fairly stable, even when exposed to light, and was diluted with 10 parts of distilled water for use. Del Rio-Hortega (1916) used a similar method for preparing the ammoniated silver bath.

Agduhr (1917) made an extensive study of the Bielschowsky method. He used many kinds of tissue and employed a great number of technical variations. He recommended the use of 8% formaldehyde for fixation and subsequent thoro washing. For the preliminary silver bath, he employed 3% silver nitrate and washed the blocks again for 1 hour in several changes of distilled water. With respect to the ammoniated silver solution, he improved the original method by measuring the size of the drops used when adding the sodium hydroxide and ammonia. He experimented with this solution by varying the amount of sodium hydroxide, and found that a reduction in the amount of sodium hydroxide produced a much lighter connective tissue stain. He also noted that, altho washing with dilute acetic after ammoniated silver would reduce the stainability of connective tissue, too much of it prevented the staining of the nerves.

Da Fano (1914) proposed a modification of the Bielschowsky method for the study of reticular tissue in the spleen, lymph glands, and other organs. In 1919

he used this technic to demonstrate the finer structure of nerve cells. This work illustrated the use of the Bielschowsky method for staining both nerve fibers and connective tissue with no apparent modification, regardless of the results which the investigator desired to obtain. In 1920, he suggested that the optimum concentration of gold chloride for toning was from 0.1% to 0.2%. He stated that the exact dilution was uncritical and that the addition of acetic acid, as suggested by Bielschowsky, was beneficial in obtaining a purplish background.

Kubie and Davidson (1928) investigated the chemical equilibria of ammoniacal silver solutions as they are employed in the methods of Fajerstajn, Cajal, Bielschowsky, and Del Rio-Hortega. They pointed out that the same complex silver-ammonia cation was produced whether sodium hydroxide, sodium carbonate, or ammonium hydroxide was used for precipitation. The activity of this cation showed striking variations, however, depending upon the constituents employed in its production. The behavior of these three ammoniacal silver solutions toward formaldehyde fixed tissues, and toward formaldehyde reduction in the test tube, was found to be identical. The solution prepared with ammonia alone was reduced most slowly and combined the least easily with the tissue. The ammoniacal silver hydroxide was reduced most rapidly and combined most readily with tissues. Ammoniacal silver carbonate stood midway between these two extremes.

Kubie (1929), in company with Del Rio-Hortega, made the observation that the strength of the formaldehyde reducing agent had considerable effect upon the appearance of sections stained with silver and ammoniacal silver reagents.

Rogers (1931) reported several section-staining methods whose essentials were the use of a 40% silver nitrate solution for impregnation and of 4% acetic acid to decolorize sections which were too heavily stained by the ammoniacal silver reagent.

The Bielschowsky method was originally designed for the demonstration of the finer structures of nerve cells and their processes, but numerous modifications of the method have been made for the study of connective tissue or reticulum. The earliest of these connective tissue methods was sponsored by Maresch (1905). Snessarew (1910) proposed a modification, which included mordanting with iron alum, for connective tissue.

Perdrau (1921) noted that connective tissue fibers were occasionally stained an intense black in material treated with Bielschowsky's method for axis cylinders.

Foot (1924) developed a similar technic for demonstrating reticular fibers in Zenker-fixed paraffin sections. Foot and Menard (1927) devised a rapid method for staining reticulum. Foot (1928), in studying the chemical differences between collagenous and reticular connective tissue, performed a series of experiments which threw some light on the various steps employed in the impregnation of connective tissue.

Guyon (1931) published some of the lessons learned from long experience in endeavoring to impregnate connective tissue with ammoniacal silver solutions. He considered the ammoniacal silver method the most complex and delicate of all histological procedures and each tissue a special problem requiring individual treatment.

Altho originally designed for neurological work, the Bielschowsky method has been used without very great modification to stain non-nervous tissue-components and particularly connective tissue. Since the literature contains a variety of results from a common procedure,

the chief problem seems to be that of securing good stains of nerve cells and processes without coincident staining of connective tissue.

Material and Methods

Spinal cord and eye muscles of adult cats, dogs, and rabbits were used for block staining by the Bielschowsky method. Spinal cord was removed from the animal within 2 hours post mortem, cut into pieces of 1 cm. or less in length, and put immediately into the fixing fluid. When partially hardened, the dura mater was split along the line of the anterior or posterior fissure, since it was found that if the dura was left intact it caused a distortion of the softer nervous tissue.

The several steps of the Bielschowsky method were varied, at first independently, then conjointly; but a block treated according to the method as outlined by Bielschowsky (1909) was used as the standard of comparison for each variation. Thus, if the fixation was varied, the succeeding steps followed the control method; later, two or more variations were introduced into the same procedure, when it had been determined that one of them exerted a definite and unchanging effect. The control method was as follows:

Fixation, 4% formaldehyde, 4 to 6 days.
Washing, 12 to 24 hours.
Pyridin extraction, 2 to 3 days.
Washing, 24 to 48 hours.
Impregnation, 1.7% silver nitrate, 3 to 4 days at 37° C.
Ammoniated silver impregnation, 24 hours.
Rinsing in distilled water, 5 to 10 seconds.
Reduction, 4% formaldehyde, 24 hours.
Dehydration in alcohols.
Embedding in paraffin, sectioning, and mounting.
Gold toning, 0.1% gold chloride.
Fixation, 5% sodium thiosulfate.

All blocks were sectioned at least to the center and sections from various locations, including the periphery, were mounted for subsequent study.

I. FIXATION

(*A*) *Fixatives.* The following fixing fluids were investigated:

4% formaldehyde.
4% formaldehyde 100 cc. + copper sulfate, 0.5 g.
4% formaldehyde 100 cc. + sulfosalicylic acid, 5 g.
4% formaldehyde 100 cc. + trichloracetic acid, 10 g.
4% formaldehyde 100 cc. + trichloracetic acid, 5 g.
4% formaldehyde 100 cc. + trichloracetic acid, 1 g.

4% formaldehyde	100	cc. +	trichloracetic acid,	0.5	g.
Absolute alcohol	100	cc. +	trichloracetic acid,	10	g.
4% formaldehyde	95	cc. +	glacial acetic acid,	5	cc.
4% formaldehyde	98	cc. +	glacial acetic acid,	2	cc.
4% formaldehyde	99	cc. +	glacial acetic acid,	1	cc.
4% formaldehyde	99.5	cc. +	glacial acetic acid,	0.5	cc.
4% formaldehyde	99	cc. +	glacial acetic acid,	1	cc.
			trichloracetic acid,	1	g.
4% formaldehyde	25	cc. +	½M. ferric nitrate	75	cc.
4% formaldehyde	50	cc. +	½M. ferric nitrate	50	cc.
4% formaldehyde	75	cc. +	½M. ferric nitrate	25	cc.
4% formaldehyde	90	cc. +	½M. ferric nitrate	10	cc.

In addition to the combination of fixing fluids as listed, a brief preliminary fixation in 4% formaldehyde was used, followed by sulfosalicylic and trichloracetic acid, either alone or in combination with formaldehyde for periods of from 18 hours to 4 days.

(*B*) *Fixation time.*

In general, the fixation time was from 4 to 6 days; thus, 8 series of blocks were fixed 4 days, 6 series for 5 days, and 2 series for 6 days. In a single series, blocks were fixed 2 hours, 3 hours, 7 days, and 3 months, respectively.

II. EXTRACTION

Pyridin in full strength was used for the routine extraction of all blocks with the exception of two series. In one of these the blocks were immersed in glacial acetic acid for the 2-day period allotted for extraction. Fixation, washing, and subsequent procedures were not varied when the acetic acid was used. The other series included extraction with 2% ammoniated alcohol, plain absolute alcohol, and on extraction.

III. PRELIMINARY IMPREGNATION

The following variations were undertaken to test the effect of an acid or alkaline silver bath, a high or low concentration, and reagent silver nitrate as compared with the more common U. S. P. product.

Group 1. Acidified.

8.5 % silver nitrate 50 cc. + 10 % nitric acid 50 cc.
8.5 % silver nitrate 50 cc. + 10 % nitric acid 25 cc.
1.7 % silver nitrate 50 cc. + 1 % nitric acid 50 cc.
1.7 % silver nitrate 50 cc. + 0.1% nitric acid 50 cc.
1.7 % silver nitrate 50 cc. + 0.1% nitric acid 25 cc.

Group 2. Alkalinized.

8.5 % silver nitrate 50 cc. + pyridin 50 cc.
8.5 % silver nitrate 50 cc. + pyridin 25 cc.
1.7 % silver nitrate 50 cc. + 10% pyridin 50 cc.
1.7 % silver nitrate 50 cc. + 10% pyridin 25 cc.

Group 3.

0.17% silver nitrate.
1.7 % silver nitrate.
8.5 % silver nitrate.
20.0 % silver nitrate.
40.0 % silver nitrate.
40.0 % silver nitrate, 24 hours; 20%, 24 hours; 10%, 24 hours; 1.7%, 24 hours.

Group 4.

Reagent silver nitrate, 1.7%.
U. S. P. silver nitrate, 1.7%.

IV. AMMONIATED SILVER IMPREGNATION

The ammoniated silver solution was made up in the following manner for all control experiments and likewise for all experiments in which the ammonia concentration was later varied. Silver nitrate (0.5 N.) 8.5%, was used as a stock solution. Sodium hydroxide (2.5 N.) was added in an equal molecular concentration: i. e., if 100 cc. of silver nitrate were used, 20 cc. of sodium hydroxide were added. The precipitate produced was redissolved with concentrated ammonium hydroxide (Sp. Gr. 0.900), added drop by drop until the solution attained a balanced or colloidal state. It was found that this equilibrium was not difficult to obtain inasmuch as a single drop of excess ammonia produced a clear solution which stood in decided contrast to the more cloudy colloidal solution. This stock solution was filtered and diluted with distilled water to make the final solution used for impregnation 0.1 N in terms of silver, or the equivalent of 1.7% silver nitrate.

The effect of excess ammonia in the above stock solution was determined by adding 1, 2, 4, and 8 cc. of concentrated ammonia water per 100 cc. of balanced ammoniated silver. Thus a series of five trial blocks were represented by one treated with balanced ammoniated silver without excess ammonia and one each containing 1, 2, 4, and 8 cc., respectively. These excesses in terms of molalities (Ag = 1) were approximately 1.5, 3, 6 and 12 M assuming the strength of the ammonia 15 M.

Nineteen series of blocks were tried.

V. PRECIPITATION OF SILVER OXIDE IN THE PREPARATION OF THE AMMONIATED SILVER BATH

All tissue specimens were treated with ammoniacal silver hydroxide: i. e., a silver bath in which sodium hydroxide was used to precipitate the silver nitrate and ammonia to redissolve it, except in

experiments to test the effect of alkalinity of the ammoniated silver bath. Since Kubie and Davidson (1928) have described the preparation of ammoniated silver solutions with the use of sodium carbonate and ammonium hydroxide as a means of varying alkalinity, we thought it desirable to try the less alkaline ammoniated silver solutions obtained by carbonate or ammonia precipitation.

Pieces of spinal cord and eye muscle were carried thru the preliminary steps of the method with no variation from the standard procedure. However, the ammoniated silver impregnation was accomplished with the use of ammoniacal silver hydroxide, ammoniacal silver carbonate, or ammoniacal silver nitrate. These solutions were prepared according to the directions previously given, and differed from one another only in the reagent used to precipitate silver oxide. When sodium hydroxide was used, the solution obtained was ammoniacal silver carbonate; and, when ammonia was used, the solution obtained was ammoniacal silver nitrate.

VI. ACETIC ACID TREATMENT

Following the recommendation of Agduhr (1917), 2.5% acetic acid was used for a 24-hour period preceding formaldehyde reduction. In this procedure, the blocks were transferred to the acid bath directly from the ammoniated silver solution with but a brief rinsing (5 to 10 seconds) in distilled water. Formaldehyde reduction was then carried out in the usual manner.

VII. PYROGALLIC ACID REDUCTION

According to Foot (1928) the use of oxidizing agents such as potassium permanganate and oxalic acid previous to impregnation produced an intense connective tissue stain, while the use of reducing agents such as pyrogallic acid, ammonium bromide, and sodium thiosulfate completely inhibited the impregnation of reticular fibers. With this in mind, a 5% solution of pyrogallic acid was used as a reducing agent and the blocks were subjected to its action for 24 hours. In one series, the pyrogallic acid was used preliminary to the first impregnation with 1.7% silver nitrate. In another series, it was used preliminary to the ammoniated silver bath.

Observations and Discussion

I. FIXATION

(A) Fixatives.

We desired to test whether the addition of a heavy metal salt or organic acid to formaldehyde solution would be advantageous or

detrimental to the staining of nerve fibers. The addition of copper sulfate was used by Bielschowsky on sections to help identify myelinated fibers. The addition of ferric nitrate was suggested because this salt had been found (Sheinin and Davenport) to fix spinal cord without shrinkage of the ganglion cells.

Tissues fixed in sulfosalicylic or trichloracetic acid without formaldehyde underwent softening and some maceration in the ammoniated silver solution, hence the organic acids were usable only in combination with formaldehyde. With the exception of acetic acid, all of the above additions to the fixative were without advantage or detrimental. The addition of copper sulfate caused overstaining at the periphery and pale staining in the interior of the blocks, hence would appear to be applicable only to sections as originally used. The addition of ferric nitrate helped to prevent shrinkage of ganglion cells but also prevented the impregnation of nerve fibers in the gray matter. Addition of sulfosalicylic acid caused incomplete silver impregnation. Addition of trichloracetic acid decreased differentiation between nerve fibers and connective tissue. Addition of acetic acid in 2% concentration did no harm and appeared to enhance the staining of pericellular plexuses in the cord and terminal nerve nets in motor end plates of striped muscle. No specific inhibition of connective tissue was seen however.

(*B*) *Fixation time.*

The period of fixation has been emphasized by some authors as having a profound effect upon the results obtained. Da Fano (1919) recommended fixation for 3 weeks to 2 months. In this work a period of from 4 to 6 days was allotted for fixation, a time which is considered adequate by most investigators. In all of these blocks the results varied only within the limits to be expected from tissue differences. The blocks which were fixed for 3 months were very heavily stained (Figs. 1 and 2). Fixation for 2 hours produced usable sections in which the coarse fibers were well stained and the connective tissue partially inhibited. Fine fibers appeared to be deficient, as tho some of them had been unimpregnated. Thus, it would appear that prolonged fixation has an unfavorable effect upon silver impregnation in a block method, and that shorter fixation periods have a less detrimental effect than has been generally believed.

II. EXTRACTION

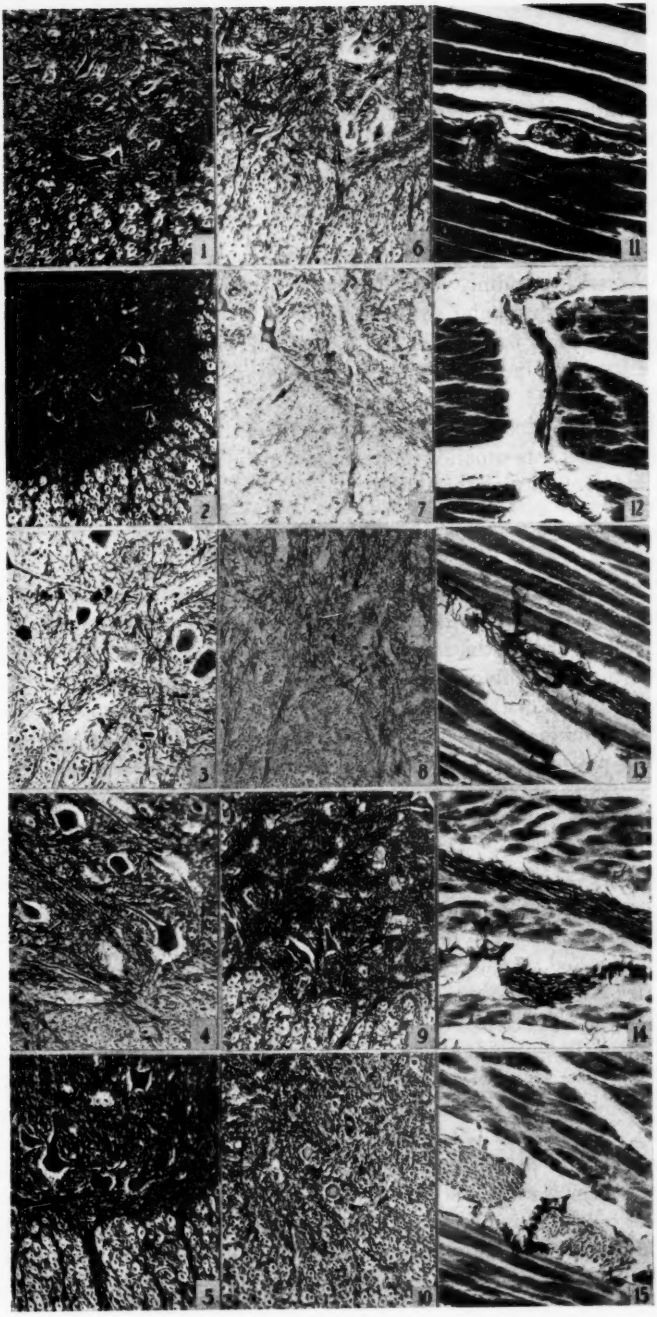
According to Weil (1929) formaldehyde fixation does not influence to any extent the galactolipids (cerebrosides) in nervous tissue. Thus,

PLATE I

1. Fixed 4 days.
2. Fixed 3 months.
3. Impregnated in strongly acidified silver nitrate.
4. Impregnated in weakly acidified silver nitrate. (This picture is also similar to that produced by impregnating in 0.17% silver nitrate.)
5. Impregnated in 40% silver nitrate 4 days.
6. 40% silver nitrate 1 day, 20% 1 day, 8.5% 1 day, 1.7% 1 day.
7. Ammoniated silver solution prepared with ammonium hydroxide only.
8. Sodium carbonate used to precipitate the silver.
9. Sodium hydroxide used without excess ammonia.
10. With 6 M excess ammonia.

11 to 15. Eye muscle preparations which show the effect of excess ammonia in the ammoniated silver bath. 11. No excess.

12 to 15. 1.5, 3, 6, and 12 M excesses, respectively.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pyridin, by dissolving the galactolipids (which make up the most of the white matter) makes subsequent penetration by silver nitrate easier.

Other solvents for lipids include alcohol, ether, chloroform, and glacial acetic acid. We have tried alcohol, both 1% ammoniated and plain, and glacial acetic acid as substitutes for pyridin. Extraction with ammoniated alcohol gave results very similar to those obtained with pyridin. Alcohol alone led to heavy staining of nuclei but pale and irregular staining of fibers. Glacial acetic acid caused large vacuoles to form in the tissue and was disqualified thereby. The tissue stained rather homogeneously without differentiation of tissue elements.

Extracted blocks were penetrated much better than unextracted and pyridin was superior to other solvents altho ammoniated alcohol could be substituted for pyridin.

III. PRELIMINARY IMPREGNATION

The preliminary impregnation in silver nitrate as used by Bielschowsky (1904) was a constant step in the present investigation. It would appear that this impregnation would have a very decided effect upon the final outcome of the method because the acidity and the concentration of the silver bath would regulate the amount of silver which combined with the tissues. Thus, a variation of the combining power of the silver nitrate was attempted by varying these factors in the preliminary impregnation.

The first variation consisted of acidification of the silver nitrate solution with nitric acid in concentrations of from 50 cc. of 10% nitric acid to 25 cc. of 0.1% nitric acid per 50 cc. of silver nitrate. Preparations treated with 50 cc. and 25 cc. of the 10% acid were not silvered after 4 days impregnation. Subsequent treatment by the usual method produced a poor result, with muscle, nerve, and connective tissue unstained, poorly stained, or unevenly stained. Of the various types of tissue, the connective tissue fibers stood out the most prominently. Acidification with 50 cc. and 25 cc. of 1.0% nitric acid in 50 cc. of 1.7% silver nitrate produced a staining of both nerve and connective tissue in muscle preparations, but with no differential effect. In sections of spinal cord, the neuroglia was stained a pale yellow but the axis cylinders in the white matter and the fibers in the gray matter were incompletely stained. Nuclei were black (Fig. 3). Better results were obtained with 50 cc. and 25 cc. of 0.1% nitric acid in 50 cc. of 1.7% silver nitrate, in that the connective tissue was partially unstained in the center but rather heavily stained at the

periphery. Preparations of spinal cord were as good as any obtained, with practically complete suppression of neuroglia in both the white and gray matter, and a very slight staining of connective tissue in the dura mater (Fig. 4). Nerve fibers were all well stained and neurofibrils were distinct in many of the large motor cells.

Alkalinization of the silver bath was accomplished by the addition of pyridin. Muscle preparations which had been impregnated in 8.5% silver nitrate to which equal or 0.5 parts of full strength pyridin had been added showed the muscle fibers stained gray with very clear black striations. Connective tissue and nerve fibers were equally well stained. The addition of equal or 0.5 parts of 10% pyridin to 1.7% silver nitrate gave better results. In specimens of eye muscle the periphery was intensely stained, but the central portions exhibited a partial suppression of connective tissue staining. Sections of spinal cord showed a suppression of neuroglial staining in the white matter but not in the gray matter. Neurofibrils were distinct in some of the motor cells, while in others the stain was so intense that recognition of cellular detail was impossible.

Variations in the strength of the silver nitrate bath were made from 0.17% to 40% with the following results:

0.17%—Neuroglia unstained; fine fibers partially stained.

1. 7%—Nerve fibers and neuroglia both stained; varying degrees of connective tissue and neuroglial staining.

8.5 %, 20.0 %, 40.0 % } Increasingly intense neuroglial and connective tissue stain with increasing concentrations of the silver nitrate.

40% 1 day, 20% 1 day, 8.5% 1 day and 1.7% 1 day—Similar to 4 days in 1.7% slightly acidified. Compare Fig. 6 with Fig. 4. No difference was observed between stains produced by reagent and by U. S. P. silver nitrate.

IV. AMMONIATED SILVER IMPREGNATION

The use of an ammoniated silver impregnation is the essential procedure of the Bielschowsky method. Originally it served as the only silver impregnation but later (1909) a preliminary impregnation in silver nitrate was employed. Schlemmer (1910), Del Rio-Hortega (1916), and Agduhr (1927) have objected to the inaccuracy of the method used by Bielschowsky for the preparation of the ammoniated silver solution. Schlemmer and Del Rio-Hortega precipitated the silver nitrate with sodium hydroxide, decanted the supernatant fluid, and repeatedly washed the precipitate before redissolving with ammonia. Dilutions of this concentrated solution were used for impregnation. Agduhr used carefully measured amounts of the re-

agents and thus improved upon the accuracy of the method. In addition, he varied the amount of sodium hydroxide and discovered that a reduction in the amount produced a lighter connective tissue stain. In effect, a variation of the amount of sodium hydroxide introduced a variation in the amount of ammonia needed for redissolving the precipitate, thus a reduction in the amount of sodium hydroxide was equivalent to an increase in ammonia, if a balanced solution was obtained. Many workers have cautioned against the presence of an excess of ammonia, and Bielschowsky accused it of causing a swelling of the axis cylinders. Agduhr noticed that too little ammonia produced the same effect.

In this work the preparation of the ammoniated silver bath has been controlled by the use of measured amounts of standardized reagents. Furthermore, the additions of an excess of ammonia were made on a constant basis.

The results obtained in 19 different series were very uniform. Sections which had come from a block impregnated in a solution which contained an excess of ammonia could be distinguished by their lighter color. Furthermore, a gross gradation in color was present from the darker control sections thru the very much lighter sections which had been treated with the greatest excess of ammonia. The greatest excess of ammonia (a molar ratio of silver nitrate to ammonia of 1 to 12) at times produced a suppression of staining which in addition to connective tissue elements involved the fine nerve fibers in the gray matter. Preparations of eye muscle (Figs. 11, 12, 13, 14, 15) showed the same gradation in the intensity of the stain, but the suppression of connective tissue was not as pronounced. Peripherally, the connective tissue of all sections was well stained; centrally, the connective tissue was unstained in the specimens treated with 6 M and 12 M excess, and partially unstained in the others. Here the 12 M excess did not inhibit the impregnation of fine terminal fibers in muscle end-plates.

The mechanism of the suppression of connective tissue staining is not very clear. It would appear that the ammonia inhibits in some way the combination of silver with the connective tissue fibers. This inhibition is somewhat selective in favor of connective tissue, for these fibers are the first to drop out with small excesses of ammonia, while the nerve fibers remain well stained until too great a concentration of ammonia reduces their affinity for silver also.

Since the dissociation of any substance is inhibited by the presence of one of the products of dissociation, an excess of ammonia will depress the dissociation of $Ag(NH_3)_2^+$ and will therefore decrease the

concentration of silver ions arising from it. The comparative lack of available silver ions in the ammoniated silver solution may be the explanation for a less dense impregnation of the tissue when an excess of ammonia is present.

It is known that washing preliminary to the ammoniated silver bath causes a loss of silver nitrate by its diffusion out of the tissue. It appears that the silver nitrate leaves the connective tissue fibers first and the nerve fibers last. Schutz (1908) washed his tissue for a considerable period after the impregnation in silver nitrate and described excellent results. Since there was no washing between the two silver baths in the present method, it is possible to account for the differential staining effect only by the presence of an excess of ammonia.

Foot (1928) stated that he obtained a complete suppression of connective tissue staining by using a strong reducing agent before impregnating the material in silver nitrate. Hence, it seemed possible that the influence of the ammonia might be accounted for by its action as a reducing agent. However, later in the investigation, pyrogallic acid was used preliminary to the impregnation and no differential effect was obtained. Thus, if as powerful a reducing agent as pyrogallic acid produced no suppression of connective tissue staining, it must be concluded that the ammonia did not exercise its inhibitive function thru any reducing action.

V. PRECIPITATION OF SILVER OXIDE IN THE PREPARATION OF THE AMMONIATED SILVER BATH

As mentioned by Kubie and Davidson (1928), the various methods used for the preparation of ammoniacal silver solutions have been the subject of a great amount of controversy. Fajerstajn, Bielschowsky, Cajal, Del Rio-Hortega, Agduhr, and others have described their methods and specific claims as to selective staining effects have been made for the various methods.

Kubie and Davidson have clarified the differences existing in these ammoniacal silver solutions, and have correlated to some degree the differences in the activity of these solutions toward formaldehyde itself and toward formaldehyde-fixed material. They found that the reactions toward formaldehyde and toward tissues fixed in formaldehyde ran parallel courses, suggesting that the same fundamental laws apply to both. Thus, the resultant action of formaldehyde as a reducing agent varies according to the alkalinity of the ammoniacal silver solution used. These authors have used sodium hydroxide, sodium carbonate, and ammonia as precipitants of silver oxide from a silver nitrate solution, and the resulting solutions have been called

ammoniacal silver hydroxide, ammoniacal silver carbonate, and ammoniacal silver nitrate, respectively. They pointed out that ammoniacal silver hydroxide, being more alkaline than ammoniacal silver carbonate or ammoniacal silver nitrate, is reduced much more rapidly by formaldehyde. Ammoniacal silver carbonate, on the other hand, due to the presence of carbonate as a buffer, stabilizes the hydroxyl ion concentration during the reduction and assists in the actual reduction by preventing the action of the acids (formic and nitric) formed during the reduction. An explanation for the differences in these solutions is made on the basis of their hydroxyl ion concentration, which, in turn, is determined by the precipitant employed. The salt $Ag(NH_3)_2OH$ which is produced in the reaction dissociates somewhat with the liberation of silver ions, hence, a slight excess of ammonia is required to balance the solution. Sodium hydroxide, a strong base, increases the hydroxyl ion concentration, produces a greater degree of dissociation, and therefore requires more ammonia to balance the solution. Sodium carbonate, when used as the precipitant, does not increase the ion concentration of either the silver or the hydroxyl ions since its own hydroxyl concentration is less than that of the ammoniacal silver solution. As a result, the precipitate produced by adding sodium carbonate to silver nitrate requires no further addition of ammonia to redissolve the precipitate than does silver nitrate itself. In the light of these differences in the ammoniacal silver solutions themselves, coupled with their variable activity toward formaldehyde as a reducing agent, it would seem that there should be some demonstrable differences when these solutions are applied to tissues which have been fixed in formaldehyde and later reduced in the same solution. Any effects may be due to one or two variables: first, a variation due to the ammoniacal silver solutions themselves; and second, a variation due to the reaction of the ammoniacal silver solution with the reducing agent.

The strength of the reducing agent and the rate of reduction of silver in tissue has produced widely variable results. It has been found that a solution of ammoniacal silver carbonate will inhibit the effect of acid formed during the reduction process, thereby enhancing and stabilizing the reduction. Ammoniacal silver hydroxide is more alkaline and produces a very rapid reduction. Thus, it has been claimed that the ammoniacal silver carbonate is more useful and produces better results than does ammoniacal silver hydroxide. However, in this work, it has been shown that an excess of ammonia in the ammoniacal silver hydroxide may be beneficial; therefore an apparent

disagreement exists concerning these findings. On the one hand, ammoniacal silver carbonate is supposed to produce good results by stabilizing the reduction process, while, on the other hand, ammoniacal silver hydroxide, which hastens the reduction in itself, produces better results when an excess of ammonia is present. An excess of ammonia, while it increases the alkalinity and therefore the rate of reduction, at the same time provides a readily accessible amount of ammonia to combine with the silver as it diffuses out during the reduction process. In this manner, the silver is kept in solution instead of being left in a form which can be readily reduced in the tissue. The ammoniacal silver carbonate appears to accomplish the same result in a different manner. The reduction process is slower, the silver has more time to diffuse out, and, finally, less silver remains in the tissues to be reduced. Therefore, the desirable effect is produced on the one hand by a combination of ammonia with the silver salt which is diffusing out of the tissue, and on the other by a slower reduction process which allows more time for the silver to diffuse out.

The results actually obtained with the three ammoniacal silver solutions agree with those postulated in the foregoing discussion. Blocks of spinal cord treated with ammoniacal silver nitrate showed suppression of connective tissue but also suppression of large nerve fibers, particularly in the center of the sections. Thus, in the white matter, except at the periphery, large fibers were completely unstained. In the gray matter small fibers predominated, but some large fibers and cells were stained (Fig. 7). Ammoniacal silver carbonate produced somewhat better results in that all the nerve fibers, both large and small, were well stained, especially in the white matter. Connective tissue staining was partially suppressed thruout. In the gray matter, the fibers were fairly well stained but the cells were so lightly stained that no cellular detail was shown (Fig. 8). Ammoniacal silver hydroxide produced a more intense stain than either of the other methods, with both nerve and connective tissue fibers prominent. Cells were well stained—in some cases too deeply stained (Fig. 9). Sections from the block treated with ammoniacal silver hydroxide plus an excess of ammonia, however, were again the best differentiated of all (Fig. 10). In these, all nerve fibers were well stained, connective tissue unstained, and cells exceptionally clear, especially in regard to their neurofibrillar structure. Thus, in a controlled series of experiments, the result produced by an excess of ammonia was most effective when used with ammoniacal silver hydroxide. Its combination with ammoniacal silver nitrate and

ammoniacal silver carbonate intensified the poorer features of the sections and did not improve the better portions.

VI. ACETIC ACID TREATMENT

In this investigation the recommendations of Agduhr have been followed and 2.5% acetic acid used for a period of 24 hours preliminary to formaldehyde reduction. The results of this series showed that the treatment tended to lighten the general tone of the sections and prevent connective tissue staining to some degree. Preparations of spinal cord were more favorably affected than those of eye muscle.

The beneficial effect of acetic acid is probably related to the reduction process since the presence of acid is known to inhibit reduction. Thus, by slowing the rate of reduction, more time is given for the silver in the tissues to diffuse out and less silver is completely reduced in the tissues than would be if the rate of reduction were rapid. The inhibition of connective tissue staining is likewise referable to the interrelation of the rate of diffusion and reduction.

VII. PYROGALLIC ACID REDUCTION

Foot found that the use of certain reducing agents produced a complete suppression of reticular tissue in his reticular and collagenous fiber staining method. The reduction was accomplished preliminary to impregnation by a combination of pyrogallic acid, ammonium bromide, and sodium thiosulfate. This idea has been applied to the Bielschowsky method by using pyrogallic acid (5%) before impregnation with silver nitrate and before the treatment with ammoniated silver. The results of these experiments were contrary to Foot's findings since the reduced material had an exceptionally heavy connective tissue stain. The sections of both cord and eye muscles were practically opaque when viewed macroscopically and only slightly translucent to microscopic observation. It must be concluded, therefore, that reduction of this nature is decidedly inadvisable in the Bielschowsky method, and that the suppression obtained by Foot was due to different conditions involved in the use of the reducing agents.

Practical Suggestions

The following suggestions were compiled in preference to any specific formulae or procedures because we believe that it is more im-

portant to know how to change a method than to attempt to give universally applicable modifications.

When preparations are too dark:

1. Treat with ammoniated alcohol after fixation instead of pyridin.
2. Use a more dilute silver nitrate bath.
3. Shorten the impregnation time.
4. Rinse longer after the silver nitrate impregnation.
5. Add an excess of ammonia to the ammoniated silver bath or use a more dilute bath.
6. Wash more thoroly with acetic acid water after ammoniated silver.
7. Use a more dilute solution of formaldehyde for the final reduction.

When preparations stain too lightly:

1. Use a stronger formaldehyde solution for fixation and lengthen the fixation time.
2. Allow the tissue to remain a longer time in pyridin.

3 to 7. The opposite of 3 to 7 above.

The addition of small quantities of nitric acid (0.01 to 0.1%) to the silver nitrate bath or of larger quantities of pyridin (0.5 to 2.5%) may be tried without fear of causing any marked impairment of the staining.

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THE FIXATION OF TISSUES VITALLY STAINED WITH TRYPAN BLUE

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ABSTRACT.—**The following fixative is recommended for tissues vitally stained with trypan blue: Chloroform, 2 parts; absolute ethyl alcohol, 2 parts; glacial acetic acid, 1 part; mercuric chloride to the point of saturation.**

The tissue should be fixed 1 to 2 hours; transferred to 95% ethyl alcohol for 12 hours; to absolute alcohol for 12 to 24 hours; to a mixture of absolute alcohol and xylol for ½ hour, and finally to xylol, before embedding in paraffin. Cedar oil may be used for clearing in the place of xylol; in that case the tissues should be transferred from absolute alcohol to a mixture of absolute alcohol and cedar oil for 24 hours before placing in cedar oil alone.

Various counterstains can be used; Mayer's carmalum is excellent.

Formalin has been the fixative most commonly used for tissues which have been vitally stained with trypan blue. Following fixation, the tissues have been prepared for sectioning either by freezing or by paraffin embedding. It is generally conceded that there is some loss of the vital dye when the tissues are treated in this manner, and that deposits of dye within the cells are somewhat less intense in color than are those in the fresh tissues. Goldmann (1909) found that formalin used as a fixative for vitally stained tissues preserved the normal contour of cells and of the deposits of vital dye better than other fixatives. He states that the amount of dye lost after fixation was insignificant when frozen sections were employed, and was only slightly greater when paraffin sections were used. In either case, he regards the loss of dye as of less significance than the distortion of the deposits which occurred when he made use of the acetic sublimate fixative employed by Bouffard (1906).

Kiyono (1914) states that there is a variable loss, from tissues during fixation, of the vital dyes used by Goldmann and that this is true not only with formalin, but also with the fixatives (i. e., bichloride and alcohol) which he himself found perfectly satisfactory for the retention of carmin. Cunningham (1922) compared fresh tissues, vitally stained with trypan blue, with sections made from the same

tissues, after fixation in alcohol, formalin or osmic acid. He found that, altho formalin preserved the contour of the cells and of their deposits of dye better than did the other fixatives, there was some loss of dye.

It is of course evident that the ideal fixative is one that will not only preserve contour, but will assure retention of even faint amounts of dye. When the type of deposition is the most important factor, the loss of a small amount of dye is of little significance, provided the form of the deposit is not distorted. For this objective, formalin has proved a most satisfactory fixative. On the other hand, when it is necessary to detect very small amounts of dye in cells which normally have a limited capacity to store it, or in which the capacity has been lessened for some reason, it is very important to be assured that no dye has been lost during fixation of the tissue and the preparation of sections. Formalin is inadequate to meet these conditions. The reports of the various workers with vital dyes indicate that the tissues retain the dyes better when fixed in alcohol or bichloride than when fixed in formalin, but suffer so much cellular distortion that the pattern of deposition is uncertain and faint traces of the dye are often obscured.

We have found that the objections to the use of formalin, alcohol or bichloride alone as fixatives for tissues, vitally stained with trypan blue, may be avoided by the use of the fixative described by Carnoy and Lebrun (1897). This fixative is composed of alcohol, mercuric bichloride, acetic acid and chloroform. It retains practically all of the dye in the vitally stained tissues. At the same time, cytological contours may be preserved by carefully modifying the proportions of acetic acid in the solution, according to the requirements of the particular tissue to be fixed. Where only one type of tissue is to be studied, little inconvenience will be experienced in the adjustment of the fixative. Where various tissues are to be fixed, the process becomes more complex. In general, shrinkage of the cells in a tissue indicates that too small a proportion of acetic acid has been used in the fixative for that tissue; swelling indicates too great a proportion. We have found that the following proportions represent the best mixture for general use:

Chloroform	1 part
Absolute ethyl alcohol	1 part
Glacial acetic acid	½ part

Mercuric bichloride to the point of saturation in the above mixture.

In order that the modifications in amounts of acetic acid to be used may be easily made, and that deterioration of the fixative due to the

formation of ethyl acetate may not take place, it has been found advisable to keep a stock supply of equal parts of chloroform and absolute alcohol saturated with mercuric bichloride, and to add the desired quantity of acetic acid to this just before use. The resulting mixture requires filtration.

The tissues should be fixed from 1 to 2 hours; transferred to 95% alcohol for 12 hours; to absolute alcohol for 12 to 24 hours; to a mixture of absolute alcohol and xylol for ½ hour, and finally to xylol preparatory to embedding in paraffin. No loss of dye occurs during embedding, and the length of time taken for embedding may be varied at will. Cedar oil may be used for clearing in the place of xylol. In that case the tissues should be transferred from absolute alcohol to a mixture of cedar oil and absolute alcohol, in which they should be left for 24 hours, and then transferred to undiluted cedar oil. They may remain in the cedar oil for indefinite periods of time.

Sections from the tissues can be stained according to any of the methods commonly used in the study of tissues vitally stained with trypan blue. We have found Mayer's carmalum (see Lee 1924, p. 137) an excellent counterstain. After being cut, mounted and dried, the sections are rapidly carried thru xylol and graded alcohols to an aqueous solution of carminic acid in alum. They are left in this from 1 to 6 hours, according to the concentration of the stain and the depth of counterstain desired. They are washed in water, carried thru graded alcohols into xylol and mounted in balsam.

We have employed this method with a large variety of normal tissues and with numerous pathological tissues, and have obtained satisfactory preservation of both vital dye and cytological contour wherever the adjustment of the acid content has been carefully carried out. The fixed tissues exhibited the same intensity of vital staining as they showed when fresh. This was true even of cells containing faint amounts of dye in lightly stained tissues, or of diffusely stained dead cells. Unfortunately, deposits of mercuric salts are always present in the sections, and as yet we have not found a satisfactory method to remove them without extraction of more or less of the vital dye, for the sole retention of which the fixative has been specifically employed. These deposits, however, should not be confused with those of the trypan blue, altho they may at times interfere with a clear view of the section, and with satisfactory photomicrography.

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NOTES ON TECHNIC

DIFFICULTIES ENCOUNTERED IN OBTAINING A SATISFACTORY WRIGHT STAIN.—For a good number of years the Biological Stain Commission has been testing Wright's stain put on the market by three or four different manufacturers, either in the dry form or the solution. Batches that have been found satisfactory have been approved and the manufacturers have been allowed to sell them under the Commission's certification label. Altho great care has always been taken in testing a blood stain before certifying it, there have, nevertheless, been more complaints made to the manufacturers or to the Stain Commission of Wright's stain than of any other stain that has been certified. Some of these troubles are probably due to the fact that for some unexplained reason certain samples of Wright's stain deteriorate in solution, while others seem to improve upon standing. The majority of the complaints, however, cannot thus be explained, but are more likely due to some fault in the technic on the part of the user. It seems well, therefore, to call attention to certain common difficulties in blood staining technic, partly for the benefit of other laboratory workers and partly in self defense against unjustified complaints made concerning certain samples of Wright's stain certified in the Commission laboratory.

Four factors are of extreme importance in securing satisfactory results with any sample of Wright's stain: clean slides or cover glasses, neutral acetone-free methyl alcohol, a correct reaction of the dilution water, and proper timing of the procedure. The importance of clean glassware and of the proper grade of methyl alcohol is to-day quite generally recognized. The other two factors, however, do not seem to have been given sufficient attention.

In such a delicate staining reaction as that involved with Wright's stain, the hydrogen-ion concentration is of extreme importance, and sometimes the procedure must be slightly modified in this respect to adapt it to special conditions obtaining in some particular laboratory. An illustration of this point is the fact that a stain which gives very satisfactory results with human blood may need some manipulation to adapt it to avian blood. In the writers' laboratory the distilled water which is usually satisfactory for staining human blood smears requires a small drop of glacial acetic acid when staining robins' blood. In many laboratories it is found desirable to employ a phosphate buffer solution (about pH 6.5) when staining human blood.

In regard to time it must be remarked that text-books ordinarily recommend one minute application of the alcoholic solution and 1 to 2 minutes with the diluted stain. With many Wright stain samples, however, a longer application is required for both steps, especially with the diluted stain where 4 to 6 minutes may be necessary. Sometimes when the red cells fail to show enough pink it may prove advantageous, besides using a more prolonged staining, to dilute the stain further than the usual proportion of 1 to 1.

Provided a certified stain is used, the methyl alcohol is of suitable quality, and the above precautions are observed, one should ordinarily have no difficulty in obtaining good results.—H. J. Conn and Lubov A. Margolena, Geneva, N. Y.

Supravital Staining of Intercellular Cement Substance.—A selective supravital staining of intercellular cement substance was recently observed by the author, while investigating the staining qualities of certain new dyes. Mention of a similar staining reaction was not found in the literature.

Frog tissue was used for the most part, tho similar staining was observed in mammalian tissues. Thin pieces of tissue, e. g., frog skin, omentum, etc., were immersed in 0.1% solution of indigo tetrasulfonate (La Motte Chem. Prod.) in normal saline, for about 10 minutes. They were then quickly rinsed in saline and placed in an 0.2% solution of methylene blue *rect. n. Ehrlich* (Grübler) in normal saline for about 1 minute. Following a quick rinse in saline the tissue was ready for microscopic examination. Permanent preparations can be made by fixation in 10% ammonium molybdate (12 hours), washing 2 hours, dehydrating in absolute alcohol, clearing in oil of wintergreen or xylol and mounting in Canada balsam.

The intercellular cement substance stains a purplish red and stands out clearly. The methylene blue stains the nuclei and cytoplasm a faint blue, enabling tissue recognition and orientation. Broad cellular sheets, e. g., peritoneum or subcutaneous lymph sheets of frog skin, are clearly demonstrated. Small lymph or blood channels and perivascular or perineural lymph spaces are readily followed by virtue of the staining of the intercellular cement substance of the endothelial lining.—Dudley A. Irwin, Dept. of Med. Res., Univ. of Toronto, Canada.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

GERSH, I. The Altmann technique for fixation by drying while freezing. Anat. Rec., **53**, 309–337. 1932.

Altmann's ingenious fixing method with freezing and dehydrating at low temperature (−15 to − 20°C.) in a vacuum has received little attention in spite of the excellent results which it gives. The author thinks that this technic has been disregarded simply because of lack of a suitable apparatus. He has constructed one which is described and pictured in this article.

Excellent cytologic fixation has been thus obtained for skin, cartilage, smooth muscle. liver, and pancreas, but poor results with the central nervous system. The Altmann technic has been successfully applied in an investigation of uric acid and ferrocyanide excretion, the chemical nature of intracellular constituents, and of the intracellular distribution of glycogen. The method has also been used for the preservation of vitally or supravitally stained preparations which fade in fixation or are incapable of satisfactory preservation by other means.

GEYER, EDMUND. Emulsion als Unterscheidungsmittel zwischen Kuhmilch und Ziegenmilch. Zts. Fleisch & Milchhygiene, **42**, 480–481. 1932.

Goats' milk may be distinguished from that of cows by means of a ring reaction with alizarin. Prepare a 20% solution of alizarin, (alizarin paste B extra, of the "Verein f. chemische u. metallurgische Produktion" Aussig). Transfer 2 cc. of it to a 25 cc. graduate cylinder, and fill up with 25% HCl, shake well and transfer to bottle. Take 20 drops of the above and add slowly some 4% sodium carbonate. Alizarin chloride is dissolved with the evolution of carbonic acid, the solution becoming red. This slightly alkaline solution prevents the curdling of milk. Add 20 to 25 cc. of 68% wood alcohol and keep well covered.

Shake the solution before use. Add 2 cc. to 5 cc. of the milk, mix thoroly and let stand. Goats' milk shows a dark blue ring on the wall of the tube, while cows' milk shows a diffuse red on the surface. A stained sediment appears in goats' milk, but is absent in that of cows. The ring appears within 3 minutes and is complete after 30 minutes. The sediment is formed within from 5 to 24 hours.

GROSS, W. and LOHAUS, H. Untersuchungen über die Wirkung von Härtungsflüssigkeiten auf tierische Gewebe. Zts. Wis. Mikr., **49**, 168–190. 1932.

The concentration and pH of various substances used for fixing and hardening of tissues are discussed in their relation to their action upon cells. A table giving quantitative data of the effects of the various solutions is given. A fluid of appropriate osmotic pressure is proposed which causes a minimum amount of shrinkage and is a good penetrant.

Dissolve 12.404 g. boric acid in 100 cc. normal NaOH and dilute with 900 cc. of H_2O. Mix 480 cc. of N/10 HCl with 520 cc. of the above described solution. Adjust to pH 7.6 with the alkaline boric acid solution or with HCl. Next add 1 g. dry potassium chromate and 1 g. $CaCl_2$ to 100 cc. of the solution.

Add 3.5 cc. of 40% formalin to every 100 cc. before using the fluid. The final pH should be 7.2. Filter, if a whitish precipitate forms. If sufficient liquid is used in fixing, the reaction will remain unaltered.

Various stains may be used; but the silver method is not suitable. The fixative offers advantages for the nervous system, where paraffin embedded sections show good differentiation in Weigert's procedure, if mordanted with copper acetate. Nissl's stain also gives good results. This would be impossible following acid solutions of chromates. As the reaction is slightly alkaline, there is no dissolving of Fe or Ca, and even in preparations with a large amount of blood no precipitates appear.

JASSWOIN, G. Eine zuverlässige Herstellungs- und Färbungsmethode der Häutchen des lockeren Bindegewebes. Zts. Wis. Mikr., **49**, 191–194. 1932.

The method offered for the examination of loose connective tissue shows collagen and frequently elastic fibers, also the ectoplasm of fibrocytes, the cells of the latter appearing distinct from histiocytes.

Add about 5 g. $MgCO_3$ to 100 cc. commercial formalin; shake, filter and then dilute to 12%. With as little exposure to air as possible, fix tissue in this for at least ½ to 1 hour. For small objects like mice, submerge the whole animal in the fixative and pull off the skin while therein. With sharp scissors cut a small portion (1 x 10 mm.) from the transparent surface. Transfer to a slide smeared with albumen-glycerin and let it partly dry. To prevent the strip from rolling, stroke it with a needle. Stretch the skin carefully but rapidly with needles so as to obtain a very thin layer. The softness of the skin determines the success of the operation. The section should adhere to the slide. Grade from 95% alcohol down to distilled water.

Stain with eosin, azure, iron hematoxylin, or some other stain. For iron hematoxylin, dissolve 1 g. dye in 100 cc. distilled water by boiling slowly for 10 minutes. When the solution gets dark red, mix 5 drops with about 12 drops of 5% iron alum solution, altering the proportion according to the ripeness of the stain. Apply 15 to 30 seconds and wash under tap. If overstained, differentiate in 1.5 to 2% alum. Counterstain, dehydrate and mount.

LAIDLAW, G. F. The dopa reaction in normal histology. Anat. Rec., **53**, 399–413. 1932.

A simplified form of Bloch's dopa reaction is recommended for demonstrating active melanoblasts and myelogenous leucocytes. For stock solution, dissolve 0.3 g. dopa (an abbreviation for 3, 4-dioxyphenylalanin) in 300 cc. of distilled water. (Purchase from Hoffmann-LaRoache, Nutley, N. J., specifying "for Bloch's dopa reaction.") This reagent, when dissolved in an alkaline solution and exposed to air, oxidizes spontaneously to a black substance resembling animal melanin, if not actually identical with it. Keep the solution well corked in a refrigerator. It is usable for several weeks, or until it darkens.

For buffers, use Sorensen's M/15 KH_2PO_4 and M/15 Na_2HPO_4. Previous to cutting the sections, buffer the stock solution to pH 7.4, by adding, to 25 cc. of it, 2 cc. of M/15 KH_2PO_4 and 6 cc. of M/15 Na_2HPO_4. Filter and replace in refrigerator. Keep all glassware scrupulously clean.

Immediately after death place tissue in 5% formalin for not over 2 to 3 hours. Freeze and cut sections, rinse quickly, and immerse in the buffered dopa. Change after 30 minutes; keep at 30 to 37°C. In about 2 hours the fluid turns reddish and later sepia-brown; this marks the end of the reaction. Wash immediately in water and mount in balsam. The leucocytes and melanoblasts should be gray or black, melanin itself yellow-brown, the background colorless or pale gray.

LECLOUX, J. Recherches sur l'anthracose pulmonaire. I. Technique d'extraction des pigments anthracosiques de l'expectoration. Bull. d'Histol. Appl. **9**, 209–212. 1932.

A clear picture of pulmonary parenchyma inclusions of anthracosic patients may be obtained by the following method: Tubercular and non-tubercular anthracosic patients are used.

Separate purulent anthracosic particles from the saliva, and transfer them to beakers. Cover with a 20% alcoholic KOH solution and boil for several minutes. Cover and keep cool for 24 hours. Heat again to dissolve all the organic matter. Transfer to test tube and centrifuge. Wash the sediment with distilled water until no trace of KOH remains. Test with phenolphthalein, until the pink color disappears. Test with silver nitrate to assure the absence of chlorides.

Add 95% alcohol and pour onto a heavy filter, wash with alcohols and evaporate. The residue will contain all the iron, mineral carbon, and some silica and lime.

Smears of the untreated sputum stained with fuchsin or Giemsa and the inorganic residue are then studied. The nature of the inorganic particles is determined by histochemical methods.

LISON, L. Sur la spécifité du réactif de Schiff envers les Aldéhydes. Bull. d'Histol. Appl., **9**, 177–195. 1932.

The author calls attention to the fact that Feulgen's fuchsin sulfurous acid reaction cannot be considered specific for aldehydes, as the following substances give a similar

color change: Aliphatic ketones, certain non-saturated compounds, pyramidon, amino-oxides, bromine, and catalytic oxydants.

In other words, the reaction is in common with that of compounds having a double bond between carbon and oxygen (carbonyle type), or a double bond between carbon and carbon (ethylene type). The difference in the reaction between those types of compounds in Schiff's test is in the shade of reactivity and the substituents have considerable influence on it. Feulgen considered the purple reaction with certain aldehydes as the real reaction in contrast to the pseudo-reaction (reddish) with acetone. The writer, however, observed that the protocatechuic aldehyde gives a reaction redder than acetone. He regards as a "true" reaction any which produces a coloration different from the original fuchsin used in the preparation of the reagent (or the fuchsinic salt of the para-fuchsin leucosulfonic acid). The pseudo-reaction (regeneration of the reagent) is produced by alkalies, and by catalytic oxydants.

The author feels, therefore, that Schiff's reagent must be checked up by other tests for aldehydes, not based upon the additional capacity of the double bond.

McNamara, W. L. A rapid paraffin technic. J. Lab. & Clin. Med., **17**, 1162-1163. 1932.

The following paraffin method is time-saving and results in sections free from shrinkage or other distortions. Fix tissue in 10% formalin. Cut into pieces 2 mm. thick. Transfer to 80% alcohol for 15 minutes; 95% alcohol, 45 minutes (40 times as much solution as tissue); acetone c. p., 15 minutes; pour off and add fresh acetone (the same quantity as in case of 95% alcohol) for 75 minutes. Place in paraffin (m. p. 54°) in oven for 1 hour. Embed and section.

In removing the paraffin from the slide use only one xylol bath. Stain by one of the usual staining procedures. Blot sections after passing them thru graded alcohols. Clear with some essential oil (e. g. clove, origanum).

Merton, Hugo. Die Verwendung von Kupfersalzen zur Herstellung von Paramaecium Präparate. Arch. Protistenkunde, **76**, 171–187. 1932.

Iron or preferably copper salts are used in the fixation of *Paramecium*, and with less success on *Stentor*. Place a drop of a mature *Paramecium* culture on a well smeared (albumen-glycerin) slide; after ½ minute, add a drop of 2% copper sulfate, holding the pipette close enough to avoid unnecessary agitation, for nearly 5 minutes. Prolong the time of fixation for weaker solutions, and vice versa. Copper chloride gives poorer fixation; copper acetate is excellent in 5 minutes, 2% in 3 minutes, or 1.5% in 45 to 90 seconds; iron salts do not fix the organisms to the slide.

Pipette off the fluid and place the slide face downwards over a 2% osmic acid solution, only 1 minute if the solution is fresh. Add a few drops of saturated $HgCl_2$ for 10 minutes. Examine the preparation; if satisfactory, place in 70% alcohol, 10 minutes, then in distilled water, 2 minutes. Formol and glacial acetic vapors may also be used for fixation with slightly different treatment.

Stain with Hamburger's acid alum carmin with light green, or with Heidenhein's hematoxylin with congo red. Heidenhein's anilin blue orange G gives remarkable results (following the osmic acid fixation): the cilia, trichocysts, and cytopharynx are blue, while the rest of the body is orange. Dehydrate and clear thoroly. Mount very carefully, as the organisms become rather brittle.

By means of this method the anterior and posterior ends are clearly revealed, showing the peristomeal grove, nuclei, contractive and food vacuoles and cilia.

Patterson, J. T. A method for mounting specimens of Drosophila on microscopic slides. Science, **76**, 258. 1932.

By means of this method one may obtain slides (probably permanent) of flies that are life-like in appearance. The preparations have the advantage of permitting examinations of both the upper and the lower surfaces.

Cover the concavity of an absolutely clean microscopic culture slide (depth 1.5 mm.) with a thin layer of ever-ready mucilage or glue (Stafford's). Working under a binocular microscope arrange the fly in a desired position in the center of the cavity. The glue hardens in a few minutes and holds the fly in a definite position during the subsequent treatment. Cotton, saturated with ether, may be held just above the fly to prevent it from moving before the glue sets. Transfer the slide to equal parts of xylol and absolute alcohol for 30 minutes; then to absolute alcohol for at least 3 hours. Fill the cavity with Grübler's euparal and apply cover glass.

The specimen to be mounted should be at least 2 days old, for very young flies tend to become distorted in the alcohol-xylol mixture.

It is possible that the procedure will also prove satisfactory with other insects not adapted to the usual pinning process.

ROLLE, M. Ein Beitrag zur Kenntnis der Gram'schen Bakterienfarbung. Deut. Tierarztl. Wochensch., **39**, 65–67. 1931.

The author's conclusions about Gram's staining are as follows: The decolorization of Gram-positive bacteria is hastened by dissociated acids, alkalies and fats. The staining solution should always be freshly prepared. A picric acid solution may be used instead of Lugol's. It is desirable to indicate the length of decolorization in describing Gram-positive bacteria.

TSUCHIYA, H. A practical staining method for intestinal protozoa. J. Lab. & Clin. Med., **17**, 1163–1164. 1932.

The method described is rapid and very efficient from the standpoint of the diagnostician, for the structures of the organisms in question are clearly revealed.

Make a thin moist film out of stool and saline solution. Fix the moist film with Shaudinn's fluid at 60°C., for 10 minutes in the case of trophozoites, and 15 minutes in the case of cysts. Immerse for 10 minutes in iodized 70% alcohol. Wash in tap water for 1 minute.

Mordant in 4% aqueous iron alum for 20 minutes. Wash 3 minutes. Drain excess water off and apply Wright's stain for 1 minute, and 5 minutes diluted with distilled water. Wash in tap water for 1 minute. Dehydrate by immersing for 15 minutes in each 70%, 95%, and absolute alcohol. Clear in 2 changes of xylol, 3 minutes each. Mount in neutral balsam and leave in incubator until examined

Nuclei of various endamebae show dark blue nucleoli, chromatin granules and linin against a pink background.

VAN HEELBERGEN, TH. Färbung von Milzbrandbazillen für die Praxis nach Foth (etwas abgeänderte Methode). Deutsche Tierärzt. Wochnsch., **39**, 321–322. 1932.

A simplified modification of Foth's diagnostic staining of *B. anthracis* is recommended for practitioners as well as for students. Stain air-dried smears with Giemsa stain (Holborn) for 2 minutes. Rinse with water, dry between filter papers, pass quickly over flame and mount in cedar oil.

The organism in question stains pink, while other bacteria are blue. *B. anthracis* appears as a blue rod, surrounded by a pink capsule, if the material examined comes from a fresh autopsy. The blue staining portion decreases with age, so that material smeared several days after death shows red capsules that are apparently empty.

DOLD, H. Ein neues färberisches Einteilungsprinzip für Bakterien. Zentbl. Bakt., I Abt., Orig., **124**, 220–223. 1932.

The writer proposes a modification of the Gram stain which he claims divides Gram-positive bacteria into 3 or 4 groups. In this procedure the stain is carbol-anilin-green (3 minutes application) prepared by mixing two parts of saturated alcoholic anilin green (Strassburger's, obtained from Grübler and Co.) with 8 parts of a 1:99 mixture of concentrated commercial carbolic acid (D. A. B. 6) and distilled water. The stain is filtered and kept 24 hours then filtered again just before use. Gram's iodine solution is applied for 2 minutes, decolorization for 5 minutes follows in a mixture of one part 40% aqueous urea (Schering-Kahlbaum) with 9 parts absolute alcohol. Then counterstain (1 minute application) in a mixture of one part saturated alcoholic Bismarck brown (source not mentioned) with 9 parts distilled water.

The writer claims that Gram-negative bacteria are negative by this procedure, i. e., stained brown; but that among the Gram-positive organisms the following 3 groups can be recognized: positive (i. e., green); negative (i. e., brown); weakly positive (i. e., partly green, partly yellowish brown); while a small number show a periodical variation, being sometimes positive and sometimes negative.